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Transmissible Retrovirus in Epstein–Barr Virus-Producer B95-8 Cells

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Epstein–Barr virus (EBV) released from the B95-8 marmoset cell line has served as a prototype for biologic and biochemical studies of EBV. Here we identify and characterize a retrovirus carried by many cultures of B95-8 cells. The experiments were stimulated by the isolation of a cDNA clone from B95-8 cells in which sequences from the EBV large internal repeat were linked to *gag* sequences similar to those of squirrel monkey retrovirus, human isolate, SMRV-H. However, among 413 amino acids predicted from the nucleotide sequence of the *gag* region of the B95-8 SMRV isolate there were 48 amino acid changes that distinguished this virus from SMRV-H originally isolated from a human lymphoid cell line by Oda *et al.* (1988, *Virology* 167, 468–476). Nucleic acid and antibody probes were developed for the B95-8 isolate of SMRV. Using such probes, we found that SMRV-B95-8 was readily transmissible, independent of EBV, as an infectious virus to human B and T cell lines. SMRV-B95-8 was highly fusogenic in the presence or absence of EBV. The ultrastructural appearance of the B95-8 retrovirus was characteristic of a type D retrovirus. Cells dually infected with EBV and SMRV-B95-8 did not demonstrate increased levels of lytic EB viral replication. SMRV-B95-8 did not by itself cause lymphocyte immortalization or enhance immortalization by EBV. Thus SMRV-B95-8 does not contribute to the major biologic properties of the B95-8 strain of EBV. © 1995 Academic Press, Inc.

INTRODUCTION

Cotton-top marmoset (*Saguinus oedipus*) lymphocytes transformed into continuous cell lines by Epstein–Barr virus (EBV) are more productive of extracellular virus than most human lymphoblastoid cell lines (Miller *et al.*, 1972; Miller and Lipman, 1973). One such marmoset cell line, B95-8, produces high yields of extracellular EBV with an immortalizing phenotype. The B95-8 virus strain has been used to define the biologic properties of EBV, including tumorigenesis in primates (Shope *et al.*, 1973) and in SCID mice (Cannon *et al.*, 1990). Early experimental EB virus vaccines were prepared from viral glycoproteins harvested from cytoplasmic membranes of B95-8 cells (Morgan *et al.*, 1983). Virus stocks prepared from B95-8 cells have also been useful reagents in human genetics and immunology. Continuous lymphoid cell cultures established by the use of B95-8 virus have been important in cytogenetic studies and in biochemical and molecular characterization of genetic diseases. A variety of human monoclonal antibodies, including ones directed against melanoma antigens and the HIV-1 *env* gene, have been derived by means of B cell immortalization by B95-8 virus (Kirkwood and Robinson, 1990; Robinson *et al.*, 1990). The B95-8 EB viral genome is the only EBV genome whose complete nucleotide sequence has been determined (Baer *et al.*, 1984).

During an investigation of the mRNAs synthesized by the large internal repeat of EBV DNA we isolated a cDNA clone from B95-8 cells in which EBV sequences were found to be fused to those of a previously characterized squirrel monkey retrovirus, SMRV-H (Oda *et al.*, 1988). In the studies reported here we pursued several questions raised by this unexpected observation. Did the retrovirus sequences in B95-8 cells represent a fully transmissible infectious agent? What was the similarity between the retrovirus in B95-8 cells and the fully sequenced variant, SMRV-H? Did the retrovirus contribute to any of the previously described biologic properties of EBV?

MATERIALS AND METHODS

Cell lines and cell culture

B95-8, W91, and FF41 are EBV-producer marmoset cell lines (Miller *et al.*, 1972,1976; Fischer *et al.*, 1981). BJAB is an EBV-negative B lymphoma cell line (Menezes *et al.*, 1975). BL2, BL30, and BL41 are EBV-negative Burkitt lymphoma (BL) cell lines (Calendar *et al.*, 1987). BL30 cells were converted to carriage of EBV by infection with P3HR-1 strain HH543-5 (clone 5) (Rabson *et al.*, 1983). The converted cells were subcloned; clone 10 and clone 30 were used. BL41 cells were converted to carriage of EBV by infection with P3HR-1 strain HH514-6 (clone 16)

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. U23805.

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or with B95-8 virus. The B95-8-converted BL41 cells were subcloned; clones 14 and 21 were used (Rooney *et al.*, 1989). Raji is an EBV-positive Burkitt lymphoma cell line (Pulvertaft, 1964). X50-7 is a lymphoblastoid cell line transformed by a B95-8 derivative (Wilson and Miller, 1979). H9, JM, and Jurkat are human T cell lines (Popovic *et al.*, 1984; Weiss *et al.*, 1984). HeLa and COS-1 are epithelial cell lines (Scherer *et al.*, 1953; Gluzman *et al.*, 1981). NN912 is a BL41-derived cell line that carries SMRV-B95-8 but not EBV.

Lymphoid cell lines were grown in suspension at 37° with RPMI 1640 medium supplemented with 8% fetal bovine serum. Epithelial cell lines HeLa and COS-1 were cultured with MEME medium supplemented with 6% calf serum.

cDNA library screening, cloning, and sequencing

B95-8 cells were treated with 20 ng/ml TPA and 3 m*M* butyric acid for 1 day before total cellular RNA was extracted. cDNA was made from poly(A)$^+$ RNA, which was selected on an oligo(dT) cellulose column (Pharmacia). cDNA fragments with *Eco*RI–*Not*I adapters were inserted into *Eco*RI-digested λgt10 vector. The ligation mixture was packaged with "gigapack" extract (Stratagene). One million independent plaques were screened by nucleic acid hybridization with an *Acc*I–*Pst*I subfragment of *Bam*HI W of Epstein–Barr virus DNA. Phage DNA from positive clones was digested with *Not*I and subcloned into the pBluescript plasmid. cDNA sequences were determined on double-stranded templates by the dideoxy chain termination method according to the procedure provided by United States Biochemicals, the manufacturer of "Sequenase." The cDNA was sequenced in both directions using a series of eight different oligonucleotide primers in the retrovirus sequence and two primers in vector sequences.

Polymerase chain reaction (PCR)

The PCR procedure was based on the protocol provided by Cetus Corporation, from which the *Taq* polymerase was purchased. One hundred nanograms of total genomic DNA was used for each reaction and amplified for 30 cycles. A cycle was 1 min each at 95°, 50°, and 74°. The following DNA oligonucleotide primer sequences were derived from the SMRV-H sequence in GenBank. The number in parentheses indicates the location of the sequence in the SMRV-H sequence.

(a) 5′-TGTCCTCGCTGCAAAAAAGGAT (2589–2610),
(b) 5′-TTGTCTAATATGCCGGGCAGAAC (3000–2978),
(c) 5′-CAGGCCAAGCTTTTGTTTGC (7305–7325),
(d) 5′-TGCTCTGTCCCGCAGAGCAAC (8785–8764),
(e) 5′-GTTCTGCAGTGAGTAGGTC (7699–7679).

Southern and Northern blotting

Southern and Northern blotting was performed following standard procedures described in Ausubel *et al.* (1987). A DNA fragment of SMRV-B95-8 equivalent to bp 2615 to 2806 of SMRV-H cloned into pBluescript plasmid was used as a probe for hybridization to detect the retrovirus *gag* gene. 5′ end-labeled oligonucleotides were also used as probes.

Expression of TrpE fusion proteins, immunization, and Western blotting

A portion of cDNA2, consisting of bp 1677 to 2262 of the SMRV-H sequence, was fused to TrpE in the pATH 10 vector (Koerner *et al.*, 1991). A TrpE–*gag* fusion protein was overexpressed in *Escherichia coli* and used to raise polyvalent antisera to *gag* in two rabbits. These antisera, called RD1 and RD2, were used in immunoblotting and immunofluorescence assays. They detected a 35-kDa protein on Western blots.

Extracellular virus particle preparation

Cell culture supernatant was spun at 5000 rpm for 10 min in a Sorvall GS-3 rotor and filtered through a 0.45-μm HA filter from Millipore Corporation. PEG 8000 was added to a final concentration of 10% with NaCl at 0.5 *M*. After overnight incubation at 4°, the mixture was centrifuged at 10,000 rpm for 10 min in a Sorvall SS34 rotor, or 7000 rpm for 10 min in a Sorvall GS-3 rotor. The pellet was resuspended in PBS, layered upon a 20% sucrose/PBS cushion, then spun at 35,000 rpm for 30 min in a Beckman SW41 rotor. This pellet was considered to be the extracellular virus preparation. To prepare virus from small volumes of cell culture, supernatant fluids were filtered and centrifuged at 20,000 rpm for 20 min in a Beckman SW28 rotor.

Electron microscopy

Cells for transmission electron microscopy were prepared as described previously (Kushnaryov *et al.*, 1985). Briefly, cells were prefixed in 2% glutaraldehyde, fixed in 1% OsO_4, dehydrated in ethanol, and embedded in Epon. Embedded cells were cut with a Reichert "Ultracut" ultramicrotome (Reichert–American Optical, Buffalo, NY), collected on 200-mesh copper grids, poststained with lead citrate and uranyl acetate, and viewed in a Philips EM-400HTG electron microscope at original magnifications of ×3,600–×28,000.

RESULTS

Isolation of a hybrid cDNA containing EBV sequences joined to retrovirus sequences

A cDNA library prepared from B95-8 cells induced into lytic gene expression with TPA and *n*-butyrate was screened with a *Pst*I–*Acc*I subfragment of the EBV large internal repeat. Six cDNAs were isolated and characterized; five of them contained contiguous sequences from *Bam*HI W. The sixth, a 342-bp cDNA clone, designated

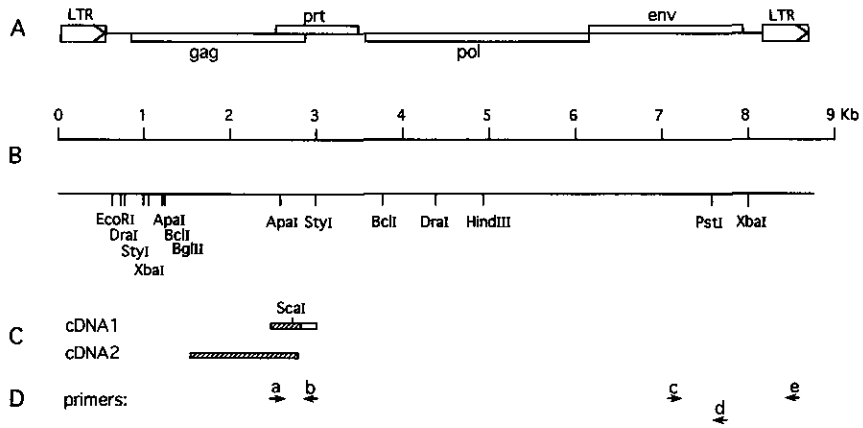

FIG. 1. Identification of the retrovirus in B95-8 cells as SMRV. (A) Diagram of the genome of SMRV-H deduced from the 8785-bp-long provirus sequence published by Oda *et al.* (1988). All the open reading frames are in one of two phases. (B) A partial restriction endonuclease map of SMRV-H. Restriction endonuclease sites that were confirmed in SMRV-B95-8 are shown. (C) Two cDNAs containing SMRV sequences isolated from B95-8 cells. cDNA1 contained both SMRV (hatched) and EBV (open) sequences. cDNA2 contained only SMRV sequences. The *Sca*I site was found in cDNA1, not in cDNA2. (D) Location of oligonucleotide primers used for detection of SMRV-B95-8. Primers a and b detect the *gag* region; primers c and e detect the *env* and LTR regions. Probe d is from the *env* region.

cDNA1 in Fig. 1C, contained 89 bp of EBV sequence from position 1624 to 1712 in *Bam*HI W linked to 253 bp of non-EBV sequence. A computerized search of the GenBank data base showed that the non-EBV sequence was nearly identical to a type D retrovirus SMRV-H previously isolated from a human lymphoblastoid B cell line of leukemic origin (Oda *et al.*, 1988). The retrovirus portion of cDNA1 corresponded to the carboxy end of *gag*, bp 2615 to 2867 in the SMRV-H sequence (Fig. 1A). In cDNA1 there were two single base changes (C to G) equivalent to positions 2678 and 2803 in the published sequence of SMRV-H. The change at bp 2803 created a *Sac*I site in cDNA1; this site was absent in SMRV-H sequenced by Oda *et al.* The point of recombination with EBV was immediately after the stop codon of *gag*.

At the recombination junction the retrovirus and EBV shared 7 bp of identical sequence, TGGGTGG; upstream of the recombination junction there was a patch of homologous sequence in which 16 of 21 bp were identical between EBV *Bam*HI W and SMRV-H. The recombination inverted the EBV sequences relative to their orientation in the conventional map of the EBV genome. No new large open reading frame was generated by the fusion of retrovirus and EBV sequences. The junction between retrovirus and EBV did not correspond to a RNA splice donor or acceptor site.

Comparison of *gag* sequences in SMRV-H and SMRV-B95-8

The cDNA library from the B95-8 cell line was rescreened with the retrovirus portion of cDNA1; cDNA2, representing bp 1677 to 2846 in the *gag* region of the retrovirus, was isolated. cDNA2 was also nearly identical to SMRV-H. Of 1179 bp sequenced there were one point mutation, seven single base insertions, and one bp deletion compared to the published sequence of SMRV-H. All these changes resulted in amino acid changes (Fig. 2). Among 413 codons sequenced in the *gag* region represented in cDNA2 there were two additional amino acids and 46 amino acid changes compared to SMRV-H. The extent of these changes, more than 10% of the sequenced region encoding *gag* protein, warrants the designation of this as a new strain, SMRV-B95-8.

The retrovirus in B95-8 cells is transmissible

To determine whether the *gag* sequences found in cDNA1 and cDNA2 represented an intact retrovirus, Northern blots were probed with the retrovirus portion of a cDNA1 (Fig. 3). A signal corresponding to a full-length retrovirus RNA genome, about 9 kb, was identified in B95-8 cells (Fig. 3A, lane 1) and also in the EBV-negative BL cell line BL41, which had been converted into EBV carriage by infection with B95-8 virus stocks. Two subclones of the converted BL41/B95-8 cells also contained full-length retrovirus RNA (Fig. 3A, lanes 8, 9). A survey failed to detect retrovirus RNA in any other cell lines carried in our laboratory. These lines included BL41 cells that had been converted into EBV carrier status by the P3HR-1 strain (Fig. 3A, lane 5) and W91 (lane 15), another EBV-producer marmoset line that carries an EBV strain from an African patient with BL, distinct from that in B95-8 cells.

The presence of genome-length retroviral RNA in BL41/B95-8 cells suggested that the retrovirus may have been transmitted together with EBV from B95-8 cells to uninfected BL41 cells. Therefore, we next determined experimentally whether the retrovirus was transmissible and whether passage of retrovirus was dependent or independent of the presence of EBV. To passage retrovirus free of EBV we took advantage of the fact that the

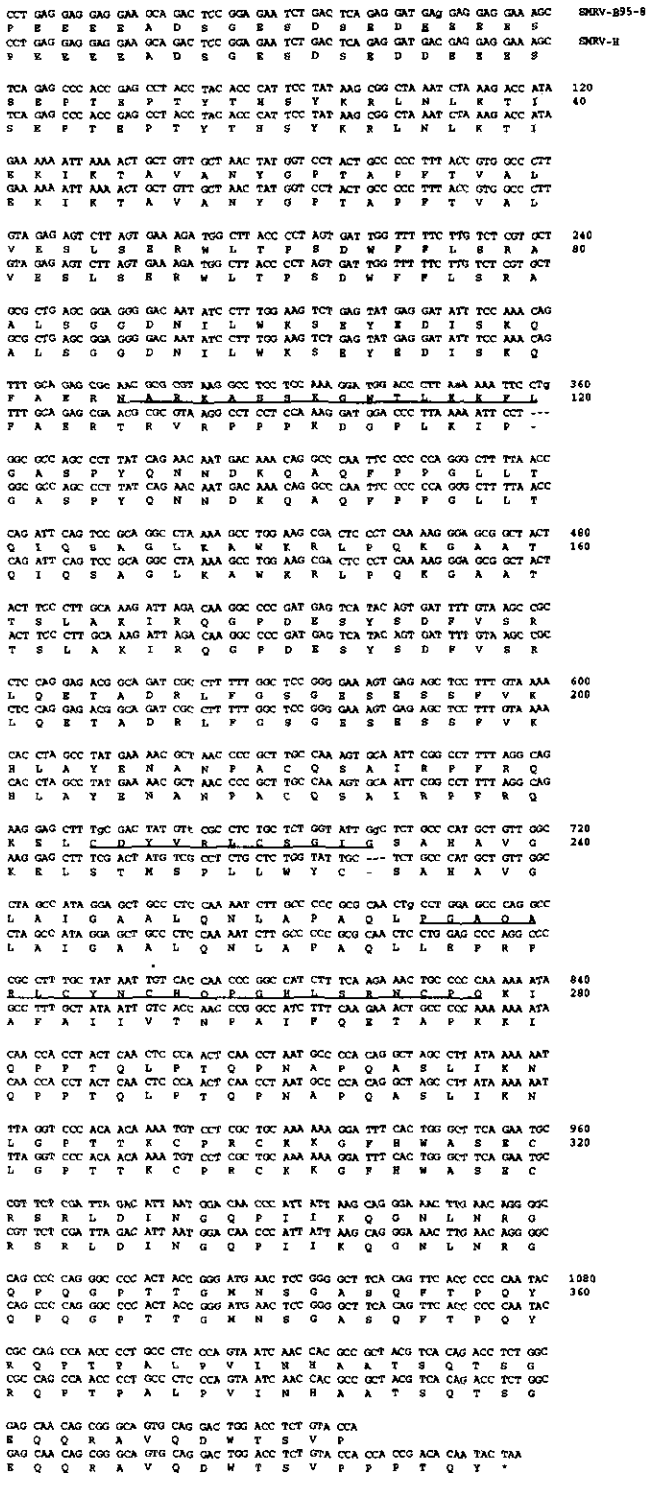

FIG. 2. The nucleotide sequence of SMRV-B95-8 cDNA2. The nucleotide sequences and predicted amino acid sequences of SMRV-B95-8 and SMRV-H are compared. Nucleotide changes in the B95-8 sequence are shown with small letters. Amino acid changes are underlined.

EBV genome in converted BL41/B95-8 is tightly latent; these cells do not release any infectious EBV (not shown). Supernatants from BL41/B95-8/Cl14 cells were added to uninfected BL41 cells. After 3 months the recipient cell line, designated NN912 (Fig. 3B, lane 2), was found to contain retrovirus RNA but lacked EBV DNA and failed to express EBV latent products when tested by immunofluorescence and immunoblotting (not shown). Therefore, retrovirus transmission does not require EBV.

In further experiments the presence of SMRV in B95-8 and NN912 cells and its transmission to other cell lines was tracked by PCR (Fig. 4). For PCR analysis one primer for *gag* was synthesized according to the published SMRV-H sequence and was not present in either of the cDNAs isolated. The other primer was present in cDNA1 and cDNA2. Other primer pairs were deduced from the published sequence of the *env* and 3′ LTR regions of SMRV-H and were not represented by any DNA clones in the laboratory. The PCR products representing the *gag* and *env* regions of SMRV-H were the same size in B95-8 cells and NN912 cells (Fig. 4A, lanes 12, 13; Fig. 4B, lanes 3, 4). This finding indicated that the virus transmitted to NN912 represented the dominant retrovirus population in B95-8 cells. Furthermore, passage of NN912 supernatant fluids or B95-8 supernatant fluids (Fig. 4, lanes 7, 9) caused various B cell lines to acquire a *gag* sequence that was represented by the same PCR product.

These experiments using PCR provided further support of the idea that SMRV-H and SMRV-B95-8 were very closely related viruses. A limited restriction endonucle-

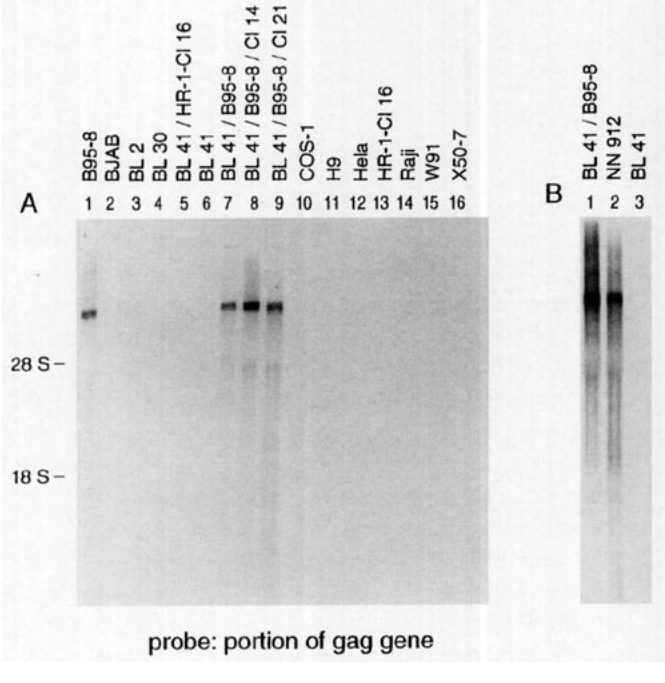

FIG. 3. Detection of SMRV-B95-8 RNA by Northern blotting. Total cellular RNA was prepared from the indicated cells. A Northern blot was probed with a *Sca*I subfragment of cDNA1 containing *gag* and vector sequences. (A) Survey of cell lines for retrovirus RNA. (B) Passage of retroviruses from supernatant fluids of BL41/B95-8/Cl14 cells to BL41 cells. BL41 cells infected with SMRV-B95-8 are designated NN912.

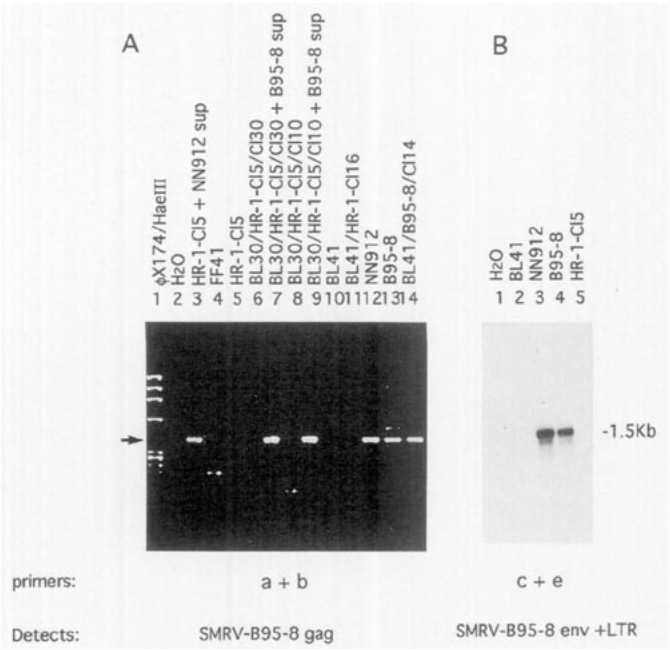

FIG. 4. Detection of SMRV-B95-8 DNA by polymerase chain reaction. (A) The primers, oligonucleotides a and b, detect the *gag* region. An ethidium bromide-stained gel is shown. Arrow indicates the expected PCR product of 412 nt. (B) The primers c and e, deduced from the published nucleotide sequence of SMRV-H, detect the *env* and 3′ LTR regions, respectively. Shown is an autoradiogram of a Southern blot probed with oligonucleotide d.

ase analysis of proviral DNA present in B95-8 cells (Fig. 1B) also confirmed the close similarity of SMRV-H and SMRV-B95-8. B95-8 cells carry two populations of SMRV. About 50% of these viral genomes contain the *Sca*I site at nt 2803.

Antibody probes for SMRV-B95-8 *gag*

To examine expression of SMRV-B95-8, polyvalent antisera were raised in rabbits to a TrpE–*gag* fusion protein that had been overexpressed in *E. coli.* Figure 5B shows that these antisera recognized a 35–kDa protein on immunoblots of cellular or virus extracts. The anti-*gag* serum was reactive with B95-8 and NN912 cells as well as with intracellular or extracellular preparations from EBV-negative BL cell lines that had been infected with virus stocks from B95-8 or NN912 cells. No signal was observed in uninfected BL cells. The preimmune rabbit serum was nonreactive (Fig. 5A).

The antiserum to p35 was used to explore the tropism of SMRV-B95-8 for human B and T cell lines (Figs. 5B and 5C). A signal representing p35 *gag,* indicating infection, was observed about 5 weeks after exposure of T and B cell lines to the retrovirus in B95-8 supernatant fluids.

Some B95-8 cultures do not contain SMRV-B95-8

In attempts to determine the origin of SMRV-B95-8, we obtained a number of B95-8 cultures from different research laboratories. Many of them reacted with the antibodies to *gag;* however, some B95-8 cultures were nonreactive. Two cultures, B95-8/CO and B95-8/EK, which did not react with the antibody to *gag* using Western blotting (Fig. 6A), also lacked the characteristic 412-bp PCR product for *gag* (Fig. 6B). The EBV DNA of the B95-8 strain has a deletion in the *Eco*RI C fragment compared with other EBV strains. The EBV genome carried in SMRV-positive and SMRV-negative cultures had the same diagnostic deletion in the *Eco*RI C region, indicating that they were *bona fide* B95-8 cells.

Electron microscopy of B95-8 cells

Two sublines of B95-8 cells were examined by electron microscopy. In one subline, B95-8/LH, which was known to contain and express SMRV sequences, mature and budding virions of type D retroviral morphology were seen in 136 of 203 cell sections examined (Fig. 7). No retrovirus was seen in the other subline, B95-8/EK, which failed to react with the nucleic acid and antibody probes. There was no difference in the frequency of productive EBV infection or in morphogenesis of EBV in the two sublines of cells. EBV nucleocapsids were seen in 3/203 profiles of B95-8/LH and in 4/314 cell sections of B95-8/EK.

Biologic effects of EBV stocks with or without the retrovirus

The presence of the retrovirus did not affect immortalization of human umbilical cord lymphocytes by EBV. In one experiment a stock of FF41 EBV, which lacks retrovirus (Fig. 4, lane 4), was titrated in duplicate. To each well of one titration an equal volume of undiluted retrovirus stock from NN912 cells was added. As a control, medium was added to the other titration. The transforming titer of FF41 EBV (78 TD_{50}/ml) was identical in both titrations. The retrovirus itself did not induce immortalization. In a second experiment B95-8 stocks with and without retrovirus (Fig. 6) were shown to transform umbilical cord cells at the same rate and to the same titer (not shown).

The retrovirus did not affect induction of lytic EBV replication. B95-8 cultures in which 85–90% of the cells were reactive by IFA with antibody to *gag* and B95-8 cells without detectable retrovirus could both be induced to express a similar level of viral DNA and late genes (not shown). The content of EBV DNA in B95-8 cells with and without SMRV was similar (Fig. 6C). A batch of HR-1 clone 5 cells that lacked het DNA was stably infected with SMRV-B95-8 (Fig. 8). An uninfected subline was handled in parallel. Neither culture expressed appreciable amounts of EBV diffuse early antigen or p21 late antigen which were evident in HR-1/Cl16 cells that had been induced into lytic cycle gene expression by butyric acid (lanes 1, 4, and 7). Reactivity with the rabbit antibody to

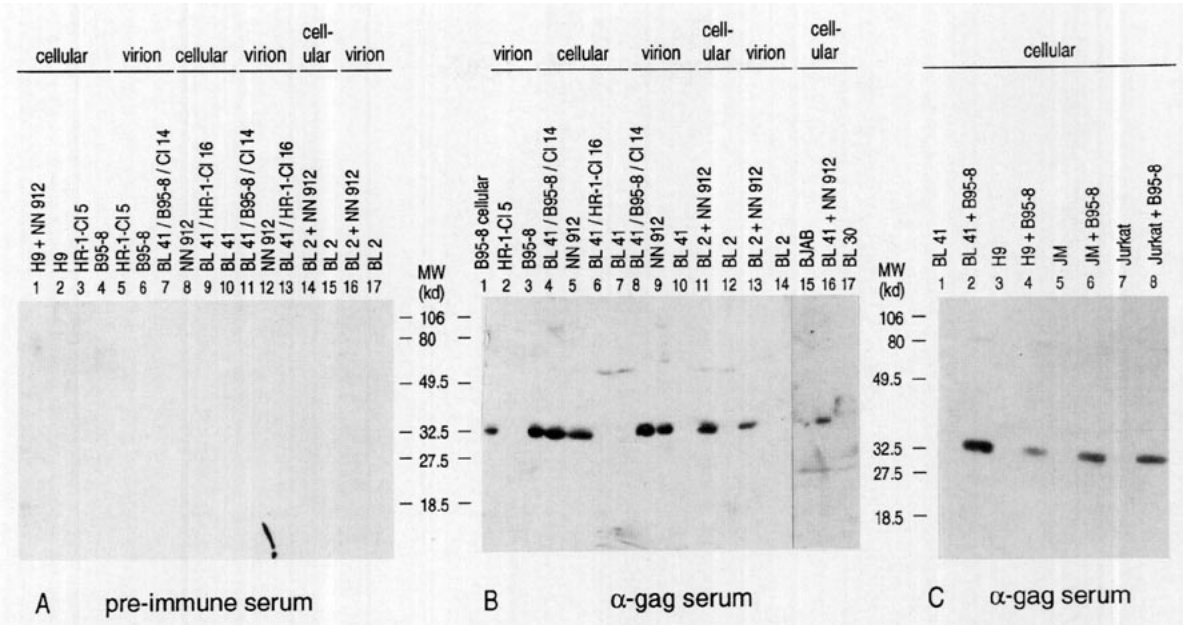

FIG. 5. Detection of SMRV-B95-8 by immunoblotting. Rabbit antibody to a TrpE–*gag* fusion protein was prepared. Where indicated with (+) the cell line was infected with frozen, thawed, and filtered (0.45 μm) supernatant fluids from B95-8 or NN912 cells. Extracts from uninfected and infected cells were prepared 5 weeks after inoculation. "Virion" extracts represent supernatant fluids that were concentrated by means of ultracentrifugation. Western blots were reacted with a 1:100 dilution of preimmunization rabbit serum (A) or with a 1:100 dilution of postimmunization serum (B and C). B shows infection of EBV-negative human B cells by SMRV-B95-8 and C shows infection of T cells.

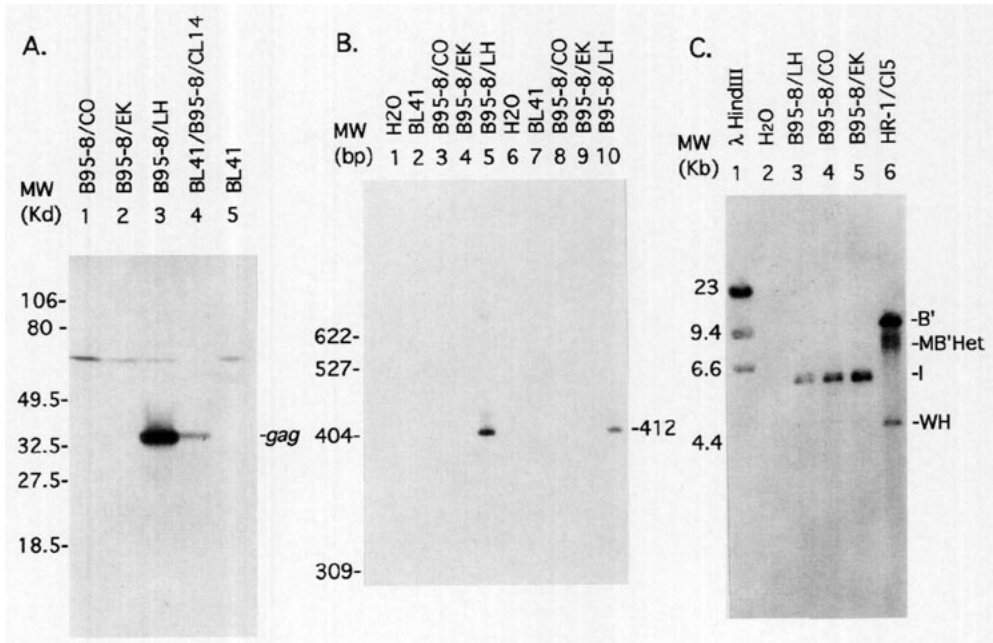

FIG. 6. Lack of SMRV in some B95-8 cultures. (A) Some cultures of the B95-8 cell lines do not express *gag* protein. B95-8/LH is the culture which was used to build the cDNA library. B95-8/CO and B95-8/EK are from different laboratories. The Western blot was probed with a 1:100 dilution of rabbit anti-*gag* serum RD1. BL41 and BL41/B95-8/Cl14 were used as negative and positive reference samples. (B) SMRV-H DNA sequences were not detected in some cultures of the B95-8 cell line. Genomic DNAs were extracted from different B95-8 cultures. Primers were based on the sequence of SMRV-H. Primer a (nucleotide 2589 to 2610) and primer b (3000 to 2978) were used for amplification of genomic DNA. A Southern blot of the PCR products was probed with the retrovirus portion of cDNA1. The experiment was carried out in duplicate from extraction of DNA to the completion of the PCR reaction. BL41 is a negative control. (C) Different B95-8 cultures carry the same diagnostic deletion in the *Eco*RI C region of EBV DNA. The genomic DNAs from different cell cultures were digested with *Bam*HI. A Southern blot was probed with the *Bam*HI B′ fragment of strain FF41. HR-1/C15 DNA is a reference EBV genome (HH543-5) which lacks the deletion in *Eco*RI C characteristic of B95-8 but contains EBV het DNA (Rabson, 1983).

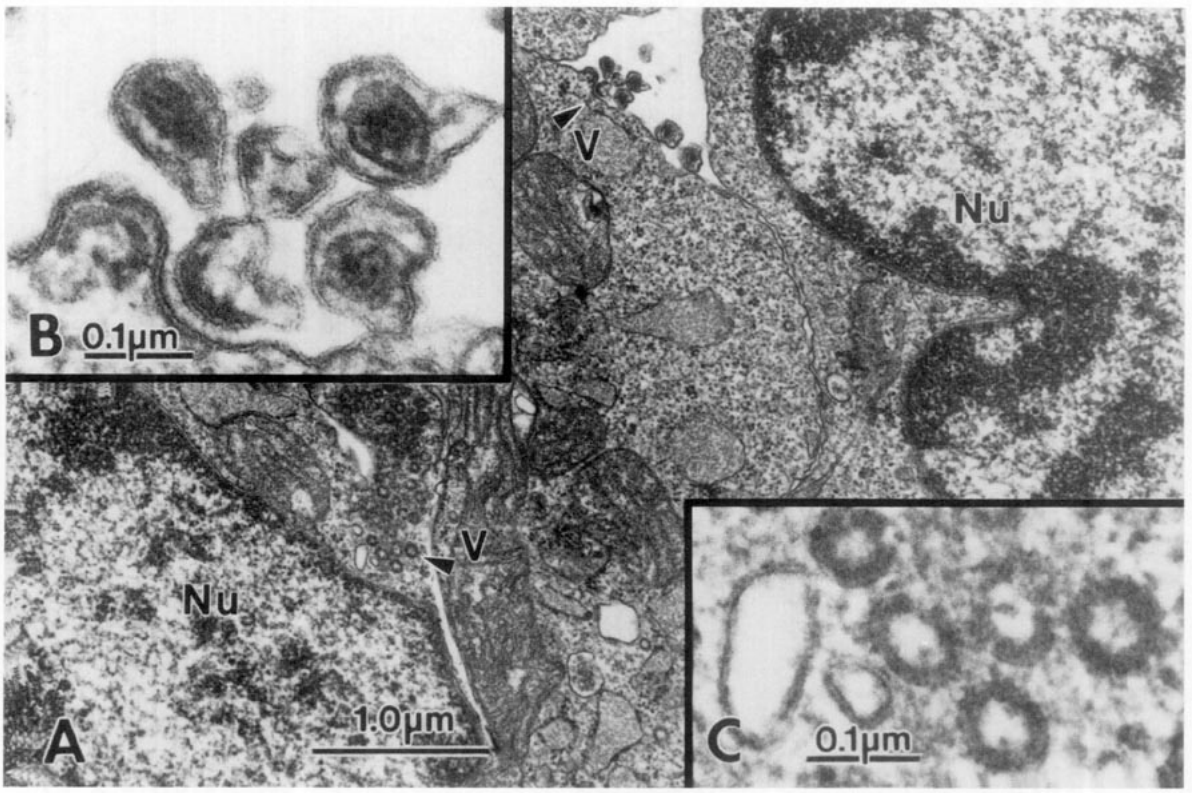

FIG. 7. Transmission electron microscopy of B95-8/LH cells. (A) Note the viral particles (V) in cytoplasm near the nucleus (Nu) as well as the free and budding virions on the same cell; (B) and (C) inserts are enlargements of sites marked by arrowheads in (A).

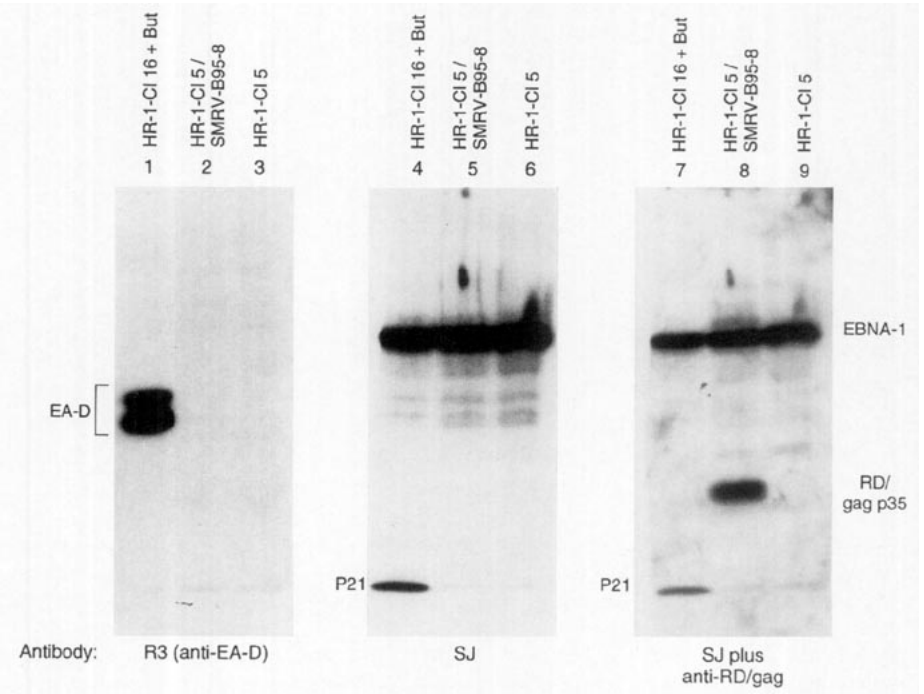

FIG. 8. Lack of effect of SMRV-B95-8 on EBV lytic gene expression. Shown are three immunoblots. These were probed with R3 monoclonal antibody to EBV diffuse early antigen (EA-D), lanes 1–3; with human antibody SJ, which detects EBNA-1 and an EBV late protein p21, lanes 4–6; with a combination of SJ and antibody to SMRV-B95-8 *gag*, lanes 7–9. HR-1 clone 5 cells infected with SMRV-B95-8 (lanes 2, 5, 8) are compared with uninfected cells (lanes 3, 6, 9). HR-1 clone 16 cells treated with *n*-butyrate serve as a positive control for lytic EBV gene expression.

gag, p35 (Fig. 8, lane 8), indicated that the HR-1/Cl5 cells had been infected.

Some virus stocks prepared from B95-8 cells have been reported to be fusogenic (Bayliss and Wolf, 1980). We found that this property seemed to correlate with the presence of the retrovirus. Supernatant fluids from retrovirus containing B95-8 virus stocks caused pronounced cell fusion in BJAB cells and less pronounced fusion in BL41 cells. Cell fusion did not require the presence of EBV. Supernatant fluids from BL41/B95-8/Cl14 or from NN912, cells that do not release EBV but do release retrovirus, induced cell fusion.

DISCUSSION

The experiments reported here were prompted by the unexpected observation of a cDNA from B95-8 cells that contains EBV sequences linked to sequences of squirrel monkey retrovirus, SMRV. The only complete SMRV genome sequence in the database is derived from an isolate made from a human lymphoblastoid cell line, designated SMRV-HLB (abbreviated SMRV-H). We show here that some cultures of B95-8 cells contain a complete, though variant, SMRV genome that represents a fully infectious retrovirus transmissible to a variety of human B cell and T cell lines. SMRV replicates independently of EBV. Conversely, EBV replication in B95-8 cells is not affected by the presence of SMRV. We have generated specific probes that permit detection of SMRV-B95-8 RNA, DNA, and *gag* protein. By means of these reagents we have shown that SMRV is not responsible for the major features of EBV biology in B95-8 cells, namely a high level of production of EBV with an immortalizing phenotype.

Retroviruses have previously been sighted during electron microscopic examination of some cultures of B95-8 cells (Tumilowicz *et al.*, 1984). On the basis of morphogenetic criteria and preference of the reverse transcriptase for Mg^{2+} rather than Mn^{2+}, these viruses have been classified as type D retroviruses. Furthermore, the *gag* proteins of these retroviruses have been found to be immunologically cross-reactive by radioimmunoassay with *gag* proteins of two type D primate retroviruses that were known to exist at the time, namely SMRV p35 and Mason–Pfizer macaque virus p26. On the basis of DNA/DNA hybridization using cDNA prepared from viral RNA, Popovic *et al.* (1982) concluded that the retrovirus present in some stocks of B95-8 cells was SMRV. Our investigations, using more specific techniques including cDNA nucleotide sequences, PCR analysis, and monospecific antibodies raised against recombinant TrpE–*gag* fusion proteins, provide a more precise identification of the SMRV variant carried by B95-8 cells.

Although there are only eight single nucleotide differences between SMRV-H and SMRV-B95-8 in a region of about 1200 bp, all these alterations result in amino acid changes. The SMRV-B95-8 *gag* protein predicted from the nucleotide sequence is at least 10% different than SMRV-H *gag*. This finding suggests that SMRV-H and SMRV-B95-8 may have different origins or that selection pressures in certain hosts favor the evolution of *gag* polymorphisms.

The origin of SMRV in B95-8 cells is still obscure. One hypothesis that can be excluded is that SMRV-B95-8 is derived from the human cell line 883L that was the source of the EBV that immortalized B95-8 cells (Blacklow *et al.*, 1971). We have found that 883L cells do not contain SMRV sequences and do not express SMRV *gag* protein (not shown). Another hypothesis is that SMRV may be transmitted to cotton-top marmosets as the result of contact between squirrel monkeys and marmosets in primate centers. As the result of such contact the lymphocytes of the marmoset used to establish the B95-8 cell line may have been infected with SMRV. This is not a frequent event, since two other marmoset B cell lines, W91 and FF41, which contain different strains of EBV, do not contain SMRV (Figs. 3 and 4). Popovic *et al.* (1982) have proposed that SMRV is a widespread laboratory contaminant of many cultured cell lines. In favor of his idea, Popovic originally identified SMRV in a myeloma cell line, RPMI 8226. Oda *et al.* (1986) isolated and sequenced SMRV from a human B cell line that originated from a patient with acute leukemia. Neither of these cell lines was said to be derived as the result of immortalization by EBV stocks prepared from B95-8 cells. However, our studies show that SMRV is not easily spread inadvertently in the laboratory. Of the many cell lines from our laboratory that were studied (Figs. 3–5), only B95-8 and B95-8-converted cells contained SMRV, and the remainder were negative for SMRV. A possible scenario for which there is no direct evidence is that in some laboratories, certain cultures of B95-8 cells acquired SMRV as the result of cocultivation with squirrel monkey lung cells (Heberling *et al.*, 1977). SMRV is an endogenous virus of squirrel monkeys (Colcher *et al.*, 1977; Hino *et al.*, 1977). SMRV sequences are present in a variety of tissues, including kidney and lung. However, SMRV is a xenotropic virus; it does not grow in squirrel monkey cells. SMRV is isolated by cocultivation of squirrel monkey tissues with human, nonhuman primate, canine, or mink cells. Therefore, the mere presence of tissue cultures of squirrel monkey origin in a laboratory is not likely to be sufficient for spread of SMRV.

Though the origin of SMRV remains puzzling, our experiments directly address some questions that have been raised previously about possible biologic synergy between EBV and the retrovirus, now definitively identified as SMRV, in B95-8 cells. SMRV does not induce EBV replication. B95-8 cultures that lack SMRV are as productive of EBV as cultures that contain SMRV. Adding SMRV to other B cells capable of being induced into lytic EBV replication does not activate a switch into the lytic

cycle (Fig. 8). SMRV by itself does not immortalize primary lymphocytes, nor does it enhance EBV-induced immortalization. Several marmoset cell lines such as W91 and FF41 that lack SMRV nonetheless release immortalizing EBV.

The studies we carried out confirm that SMRV is able to infect a wide variety of human B and T cell lines, in keeping with its classification as a xenotropic virus. Furthermore, we have isolated SMRV-B95-8 stocks that are free of EBV by passage of virus through B cells that are not permissive for EBV lytic replication. Thus we showed that long-term growth in the presence of EBV has not altered SMRV's capacity to grow independently of the herpesvirus. Nevertheless, the isolation of the chimeric SMRV–EBV cDNA raises the intriguing possibility that some infectious EBV genomes exist that contain SMRV sequences. Alternatively, some SMRV genomes may contain EBV sequences. Recombination between all or part of one virus genome with the other could change the biologic properties of either partner. The other example of recombination between a retrovirus and a herpesvirus is the integration of avian reticuloendotheliosus virus into the DNA of Marek's disease virus (Isfort *et al.*, 1992). In addition to the chimeric cDNA we have obtained other evidence for recombination between EBV and SMRV (manuscript in preparation). There is bidirectional transcription through the junction of the two viral genomes. PCR products demonstrated the presence of EBV–SMRV joint fragments in genomic DNA of B95-8 cells. The studies so far do not indicate whether these recombination events are represented in infectious virus or are present only in cellular DNA. Productive co-infection of the same cell with two different viruses offers many opportunities for recombination between the viruses, for alteration of gene expression, and for modification of cell tropism of either virus. B95-8 cells may serve as a model for EBV-retrovirus interactions.

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